Atty. Dkt. No. 081356-0233

#3

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Kazuo YAMAMOTO, et al.

Title: CARBOHYDRATE LIBRARY CONSTRUCTED BY GENE ALTERATION OF CARGO RECEPTORS

Appl. No.: Unassigned

Filing Date: 2/18/2005

Examiner: Unassigned

Art Unit: Unassigned

PETITION TO ACCEPT COLOR DRAWINGS UNDER 37 CFR 1.84

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In accordance with 37 CFR 1.84(a)(2), Applicants petition for the acceptance of color photographs to be entered in the above-captioned application.

In support thereof, Applicants submit:

I) A fee in the amount of $130.00 as is set forth under 37 CFR 1.17(h);

II) Three sets of Figures 1-16 (17 pages), all of which are in color;

III) An amendment to the specification indicating that the application contains color drawings.

03/01/2005 LLANDGRA 00000009 10525020
07 FC:1464 130.00 OP

In the Specification:

On page 1, directly beneath the title, please insert the following

--The application contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.--

REMARKS

Fig. 1 shows cargo receptors (ERGIC-53 and VIP36) involved in carbohydrate processing of glycoproteins and the quality control of carbohydrates (sugars) and the outline of secretory pathway.

Fig. 2 shows the outline of VIP36 library construction.

Fig. 3 shows the result of introducing random mutations into VIP36. "A" shows the nucleotide sequences of the putative carbohydrate-binding domains of VIP36 and "B" shows the amino acid sequences thereof.

Fig. 4 shows photographs of overexpression of altered VIP36 in MDCK cells.

Fig. 5 shows the binding of plant lectins to lectin-positive (red) and lectin-negative (black) MDCK cells. The upper row shows untransfected cells, the middle row shows mutated VIP36 transfected cells, and the lower row shows untransfected control MDCK cells.

Fig. 6 shows the binding of PHA-E4 and WGA to lectin-positive (red) and lectin-negative (black) MDCK cells. The uppermost row shows wild-type cells, the second row from the top shows mutated VIP36 transfected cells resulting from the 1st separation, the second row from the bottom shows mutated VIP36 transfected cells resulting from the 2nd separation, and the lowermost row shows the mutated VIP36 transfected cells resulting from the 3rd separation.

Fig. 7 shows the effect of trypsinization on the binding of lectins. PBS-EDTA treatment is indicated with red, and trypsin-EDTA treatment is indicated with blue.

Fig. 8 is an outline showing the design of random primers for introducing random mutations into the carbohydrate-binding domain of ERGIC-53.

Fig. 9A shows a photograph showing samples prepared using an anti-FLAG antibody as an primary antibody and a goat anti-mouse IgG1-FITC as a secondary antibody and then observed by a fluorescence microscope. As a negative control, wild-type MDCK cells were observed.

Fig. 9B shows a photograph showing samples prepared using an anti-FLAG antibody as a primary antibody and goat anti-mouse IgG1-FITC as a secondary antibody and then observed by a fluorescence microscope. As a positive control, VIP36-FLAG clone8 (clones whose constant expression has been confirmed) was observed.

Fig. 9C shows photographs showing samples prepared using an anti-FLAG antibody as a primary antibody and goat anti-mouse IgG1-FITC as a secondary antibody and then observed by a fluorescence microscope. ERGIC random libraries were transfected into MDCK cells, and then selection was carried out for 10 days with 1.5 mg/ml G418, and then the cells were observed.

Fig. 10 shows histograms showing the result of analyzing the cells following MACS screening using various biotinylated lectins as primary antibodies and FITC-labeled streptavidin as a secondary antibody. A black line indicates control MDCK cells, a red line indicates a (–) fraction following MACS, and a green line indicates a (+) fraction.

Fig. 11 shows a histogram showing the result of analyzing by FACS MDCK cells fractionated by MACS using PNA lectins. The binding strength of PNA (–), that of PNA (+), and that of PNA2 (+) to PNA were compared.

Fig. 12 shows photographs showing the results of western blotting carried out for PNA (–), PNA (+), and PNA2 (+) using 5 types of biotinylated lectins as primary antibodies and streptavidin alkaline phosphatase as a secondary antibody.

Fig. 13 shows the result of analyzing the carbohydrate-binding specificity of each cell fraction when FACS was carried out for a control, PNA (–), PNA (+), and PNA2 (+) using MAM or PNA as a primary antibody and streptavidin FITC as a secondary antibody.

Fig. 14 shows the outline of a technique for separating cells having specific carbohydrate moieties by flow cytometry or a magnetic cell sorting (MACS) using labeled lectins.

Fig. 15 shows the intensity of fluorescence for labeled PNA lectins in a process where cells having carbohydrate moieties specifically binding to PNA lectins were separated by flow cytometry (FACS) and then enriched.

Fig. 16 shows the expression of carbohydrate moieties to be recognized by altered VIP36 and PNA lectins in clone 12 and control CHO cells.

The results of these comparisons and measurements are best depicted in color and the photographs enhance visual contrast, which otherwise would be indistinct. Favorable action is solicited.

Respectfully submitted,

Date February 18, 2005

By _____

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FIG 1

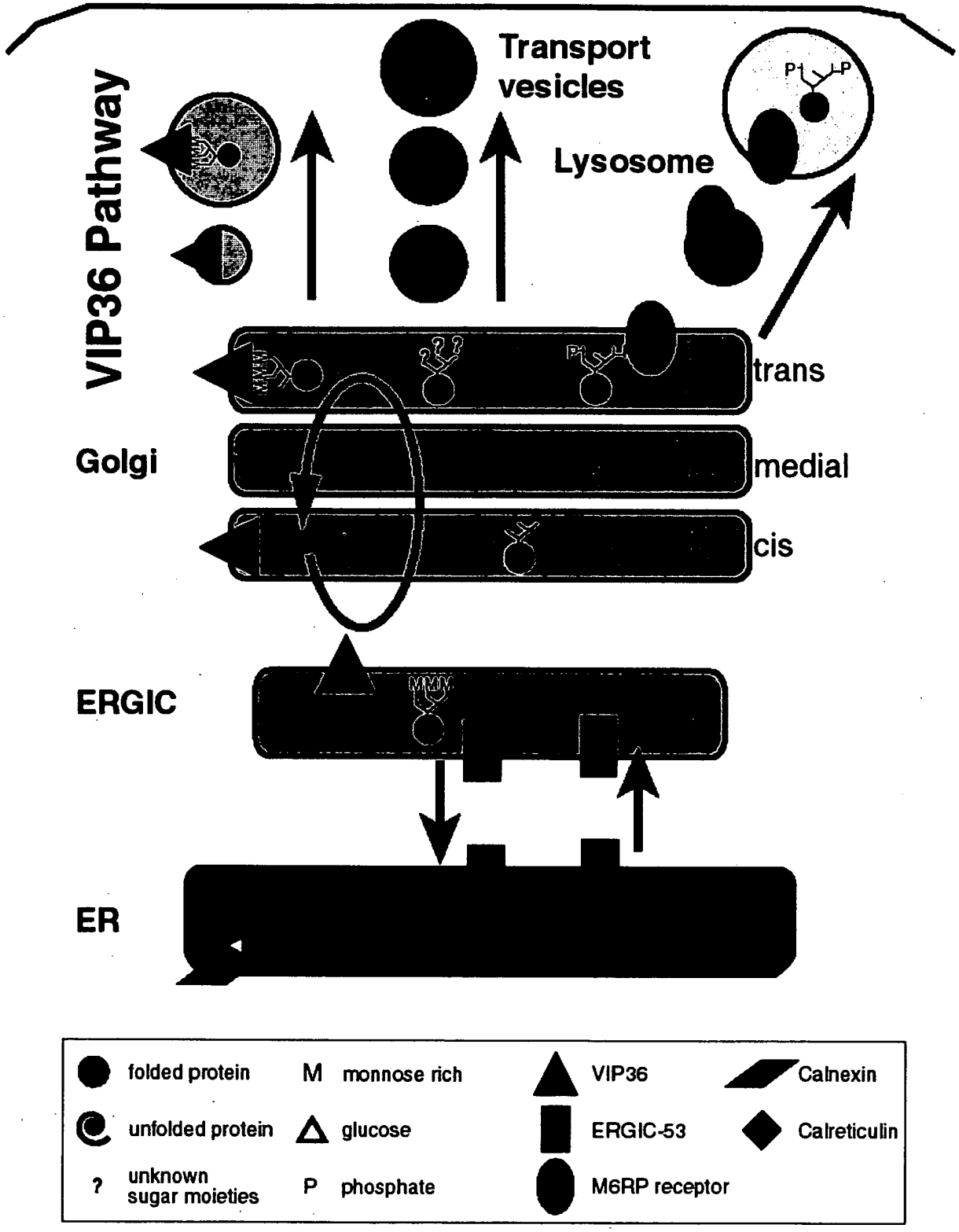

Cell Surface
Transport vesicles
VIP36 Pathway
Lysosome
P
P
trans
Golgi
medial
cis
ERGIC
ER
folded protein
unfolded protein
? unknown sugar moieties
M monnose rich
glucose
P phosphate
VIP36
ERGIC-53
M6RP receptor
Calnexin
Calreticulin

FIG 2

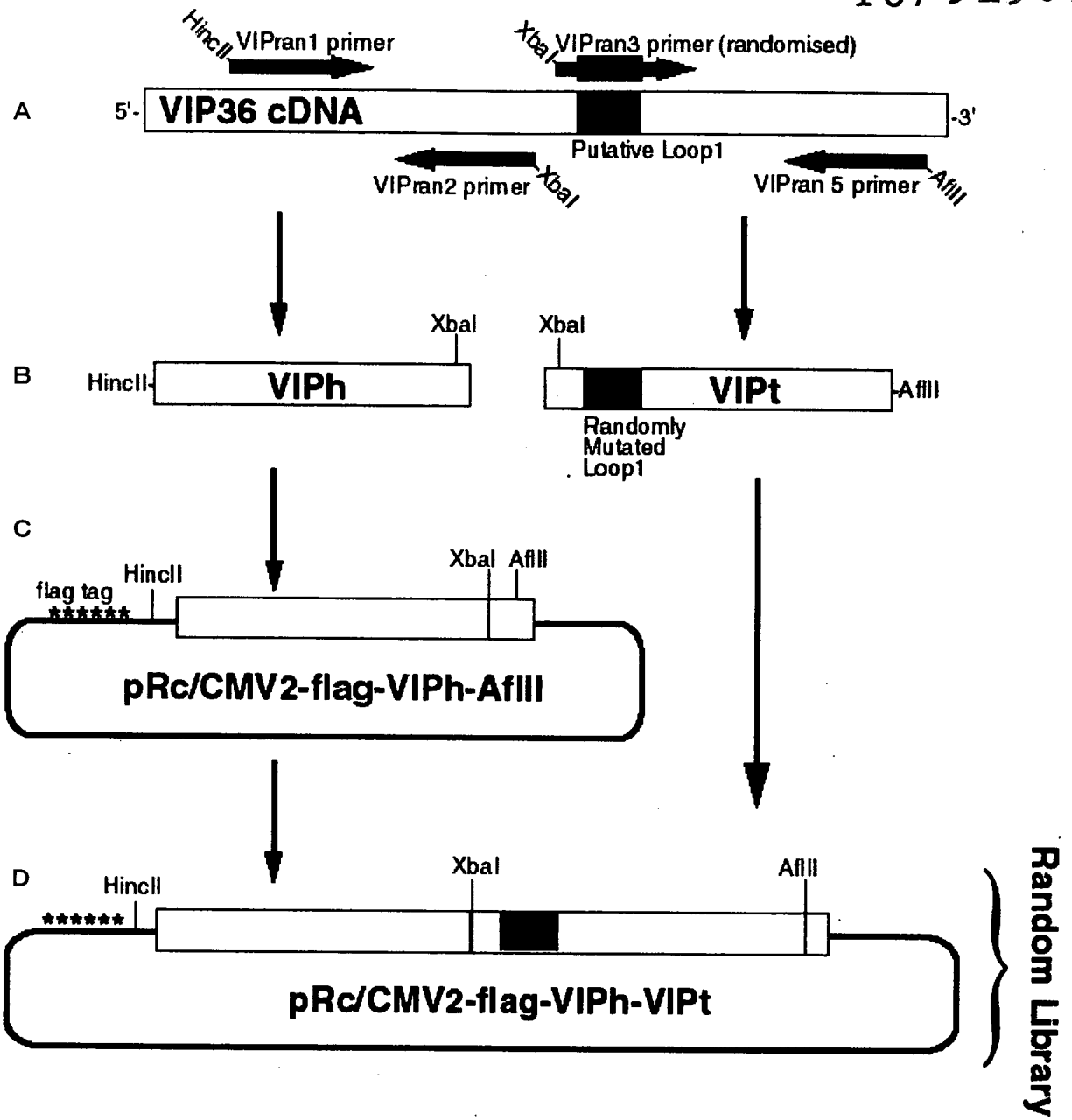

HincII
VIPran1 primer
XbaI
VIPran3 primer (randomised)
A
5'-
VIP36 cDNA
-3'
Putative Loop1
VIPran2 primer
XbaI
VIPran 5 primer
AflII
XbaI
XbaI
B
HincII
VIPh
VIPt
AflII
Randomly
Mutated
Loop1
C
XbaI
AflII
HincII
flag tag
******
pRc/CMV2-flag-VIPh-AflII
D
HincII
XbaI
AflII
******
pRc/CMV2-flag-VIPh-VIPt
Random Library

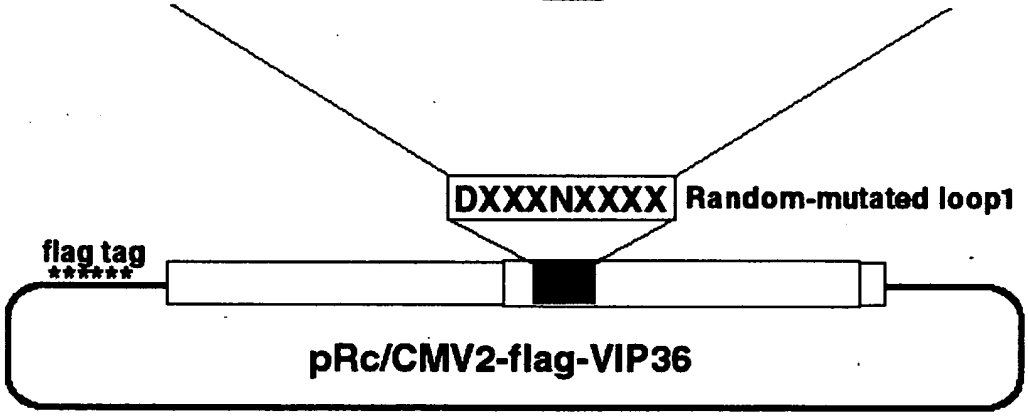
FIG 3

A

VIP36 original	GAC	ACC	TAC	CCC	AAT	GAT	GAG	ACC	ACT
vip3f clone	GAC	AGG	CAT	GGG	AAT	TAT	AGT	GTT	TAT
vip26f clone	GAC	ATT	CGT	TTG	AAT	CTT	GAG	AGT	GGT
vip28f clone	GAC	CGT	GCG	CTT	AAT	CTT	ATG	CTT	CGT
B8-VIP10.Seq clone	GAC	GTG	TTT	GCT	AAT	CAT	TGT	CAT	TTG
B2-VIP7.Seq clone	GAC	-TG	TGT	GGT	AAT	TGT	GGG	TGT	TAG
B6-VIP9.Seq clone	GAC	CAT	GGG	TTT	AAT	GCT	AAT	CGG	ATT
A3-VIP2.Seq clone	GAC	GTG	TGT	TGG	AAT	GGG	GGG	TGT	AAT

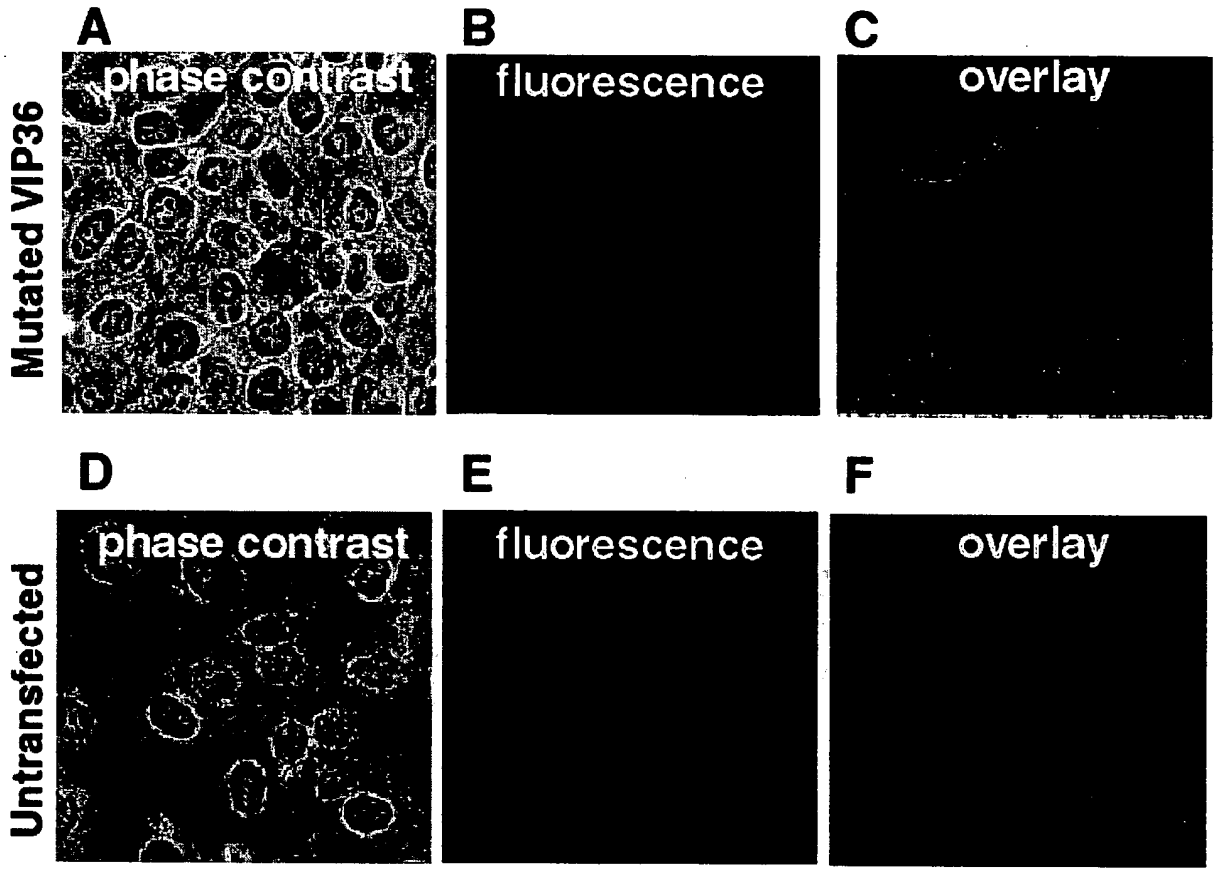
B

VIP36 original	D	T	Y	P	N	D	E	T	T
vip3f clone	D	R	H	G	N	Y	S	V	Y
vip26f clone	D	I	R	L	N	L	E	S	G
vip28f clone	D	R	A	L	N	L	M	L	R
B8-VIP10.Seq clone	D	T	Y	P	N	D	E	T	T
B2-VIP7.Seq clone	D	-	C	G	N	C	G	C	*
B6-VIP9.Seq clone	D	H	G	F	N	A	N	R	I
A3-VIP2.Seq clone	D	V	C	W	N	G	G	C	N



Constructed Random Library

FIG 4


A
B
C
phase contrast
fluorescence
overlay
Mutated VIP36
D
E
F
phase contrast
fluorescence
overlay
Untransfected

positive
negative
not separated
Log FITC Fluorescence
Relative number of Cells
untransfected control MDCK cells
no lectin + no antibody
no lectin + SA-FITC
DSA + SA-FITC
mutated VIP36
untransfected
ABA
ConA
DSA
LCA
Lotus
MAM
PHA-L4

FIG 5

FIG 6

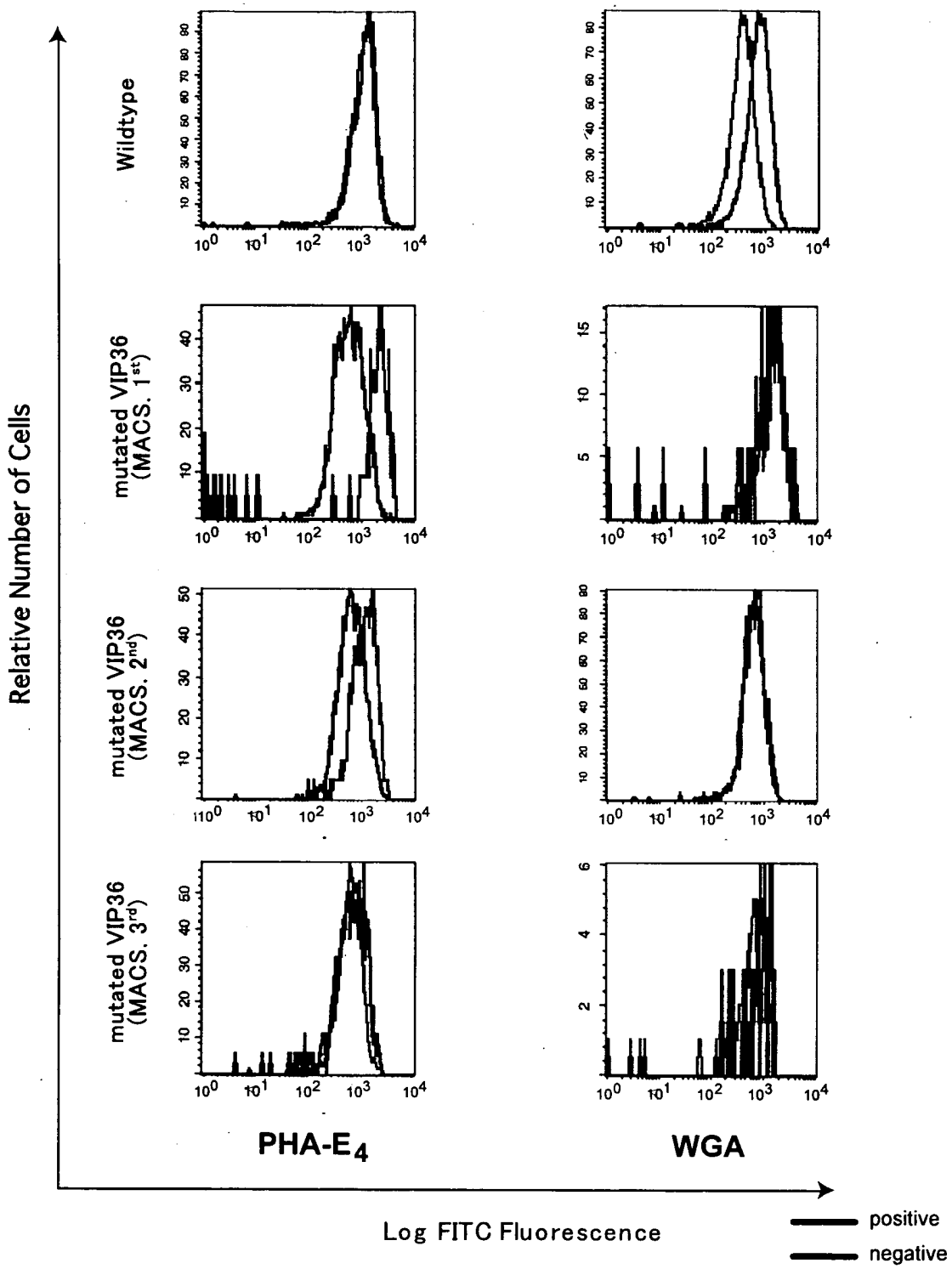
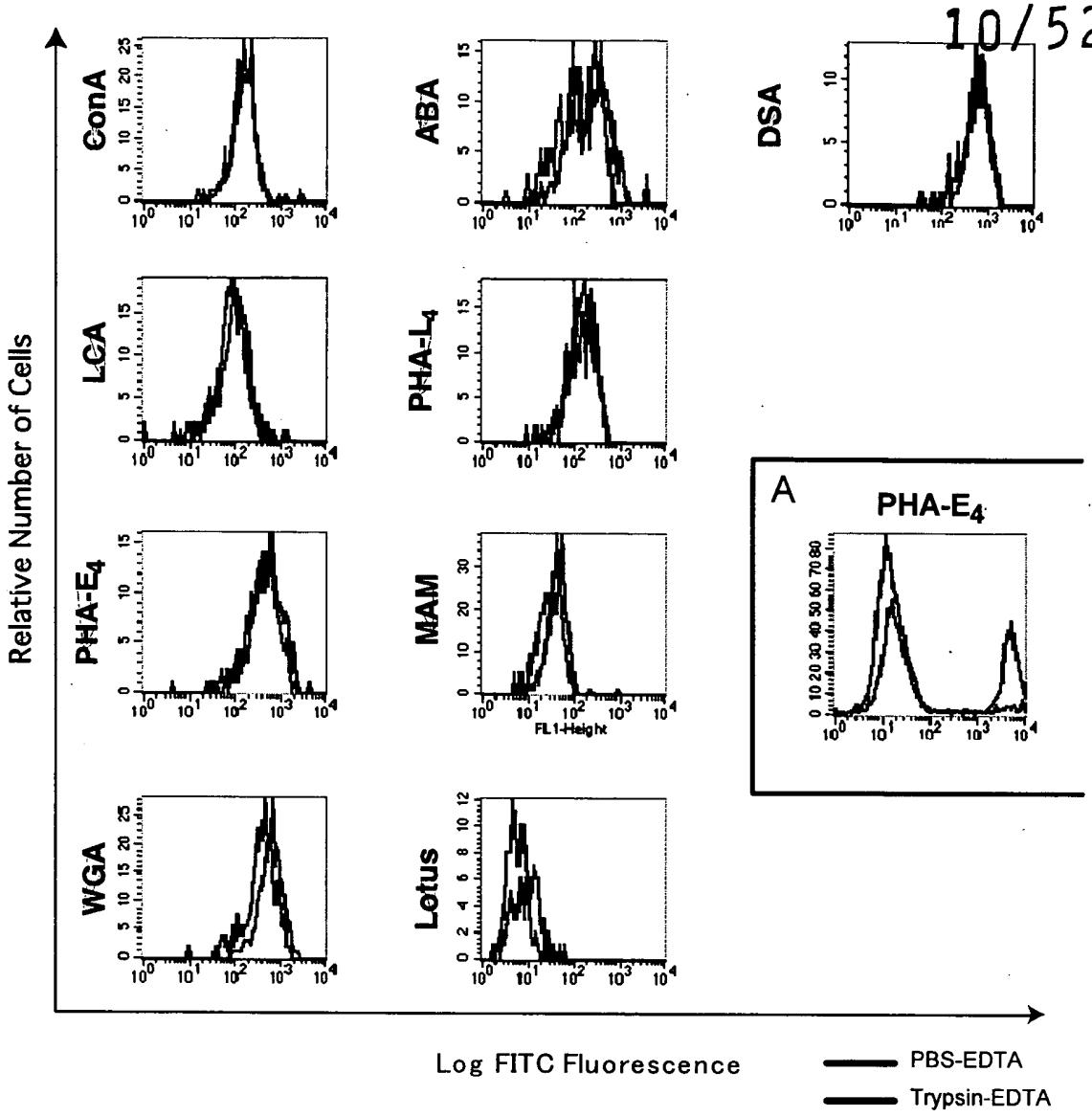

Wildtype
mutated VIP36 (MACS. 1st)
mutated VIP36 (MACS. 2nd)
mutated VIP36 (MACS. 3rd)
Relative Number of Cells
PHA-E4
WGA
Log FITC Fluorescence
positive
negative

FIG 7


ConA
ABA
DSA
LCA
PHA-L4
PHA-E4
MAM
WGA
Lotus
A
PHA-E4
Relative Number of Cells
FL1-Height
Log FITC Fluorescence
PBS-EDTA
Trypsin-EDTA

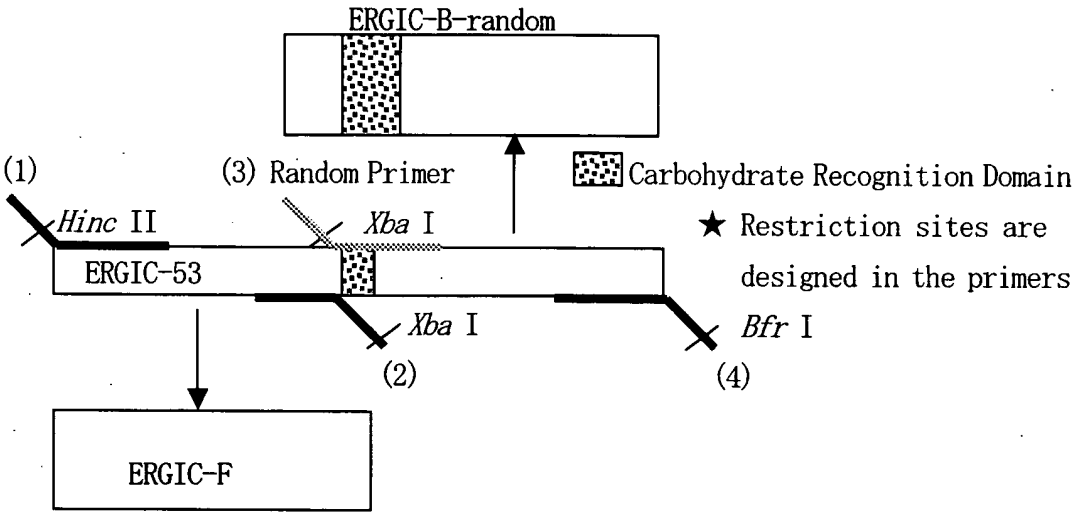

ERGIC-B-random
(1)
(3) Random Primer
Carbohydrate Recognition Domain
★ Restriction sites are designed in the primers
Hinc II
Xba I
ERGIC-53
Xba I
Bfr I
(2)
(4)
ERGIC-F

FIG 9 A

FIG 9 B

FIG 9 C

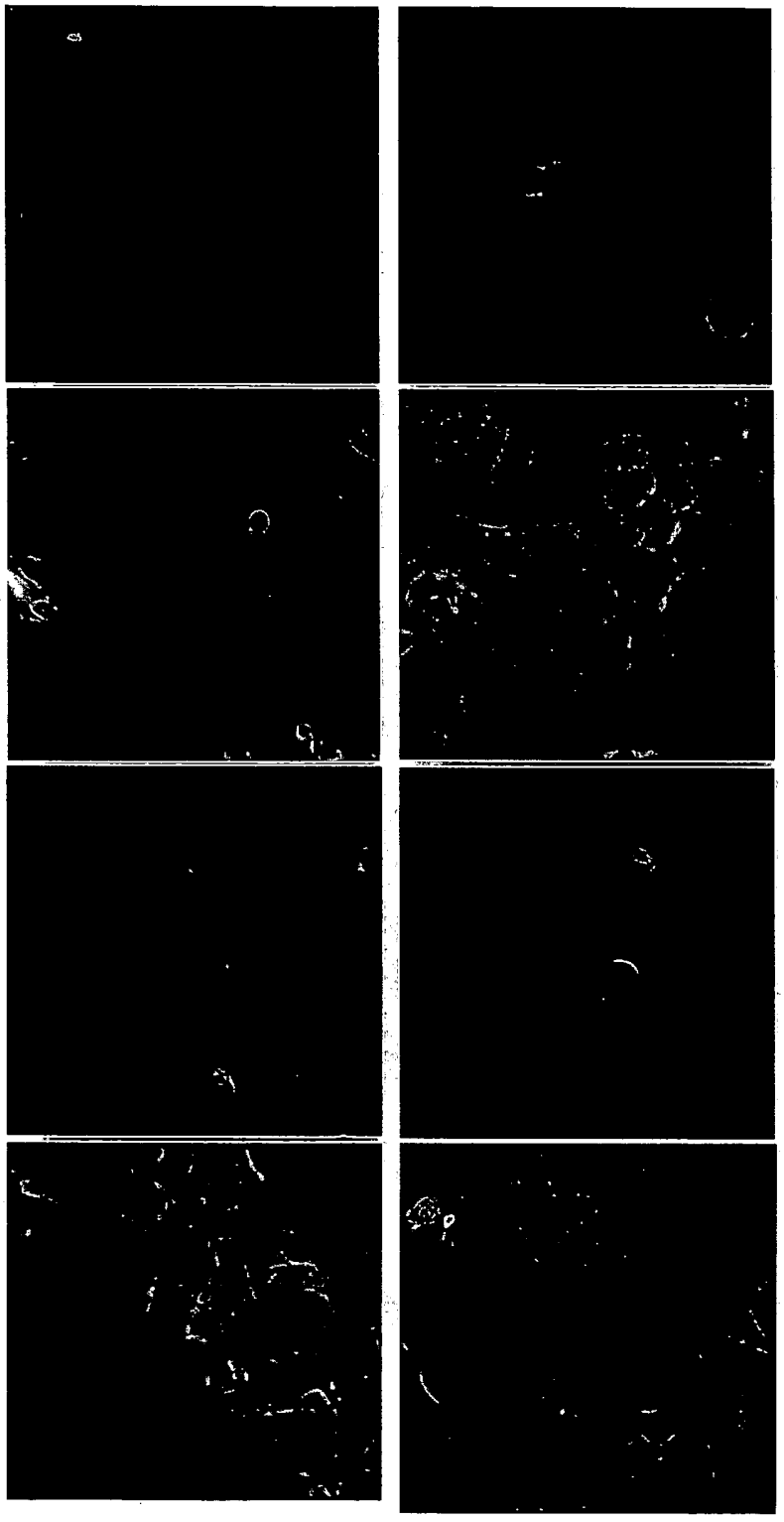


FIG 10

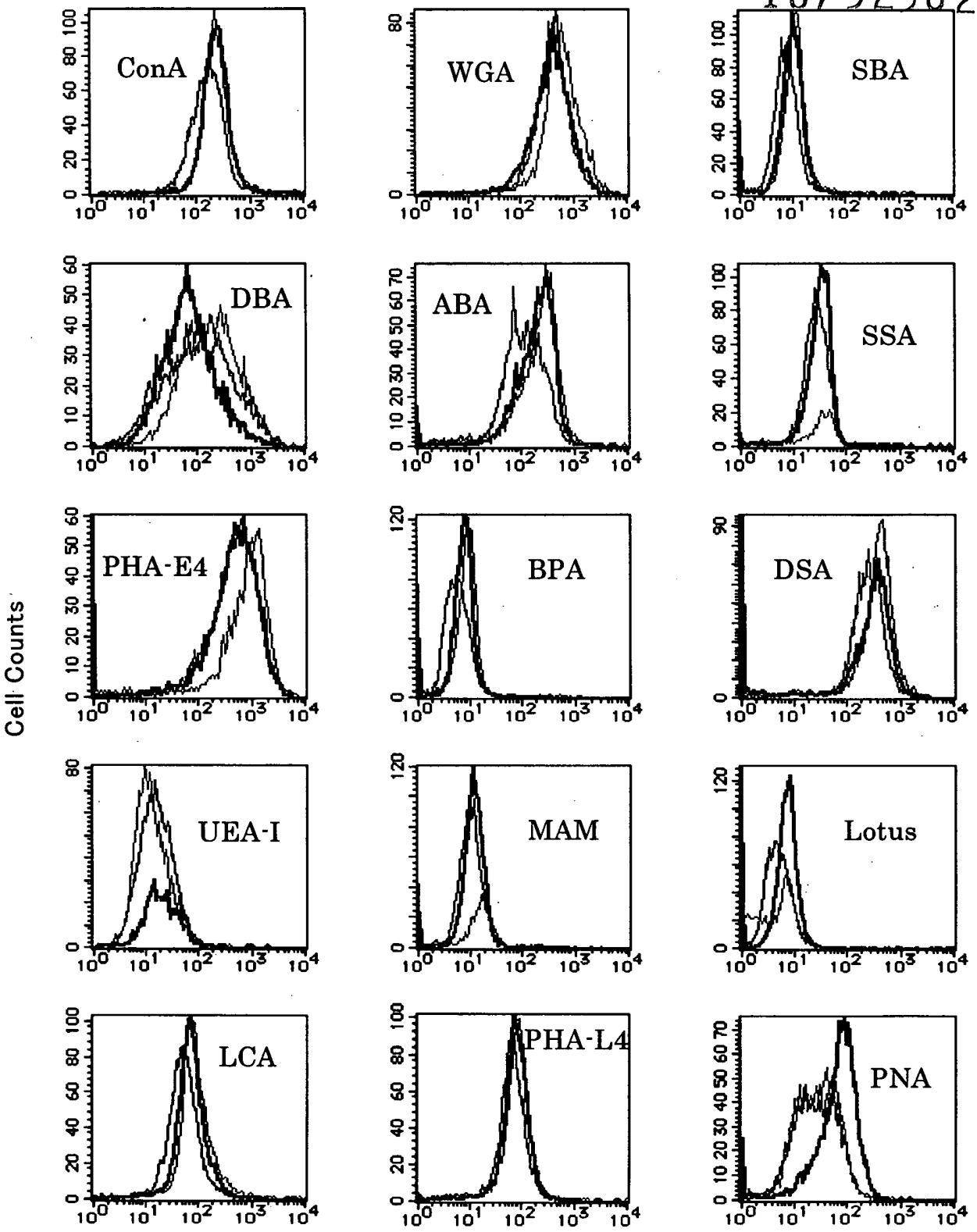

ConA
WGA
SBA
DBA
ABA
SSA
PHA-E4
BPA
DSA
UEA-I
MAM
Lotus
LCA
PHA-L4
PNA
Cell Counts
Fluorescence Intensity (log)

FIG 11

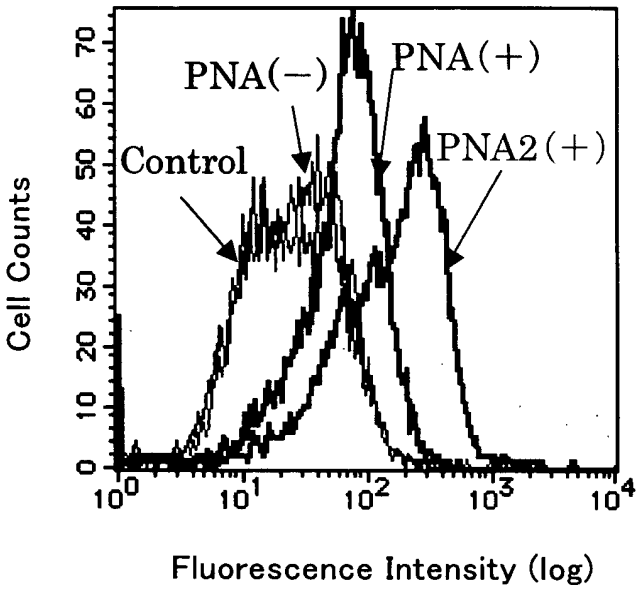

PNA(−)
PNA(+)
Control
PNA2(+)
Cell Counts
0
10
20
30
40
50
60
70
10^0
10^1
10^2
10^3
10^4
Fluorescence Intensity (log)

FIG 12

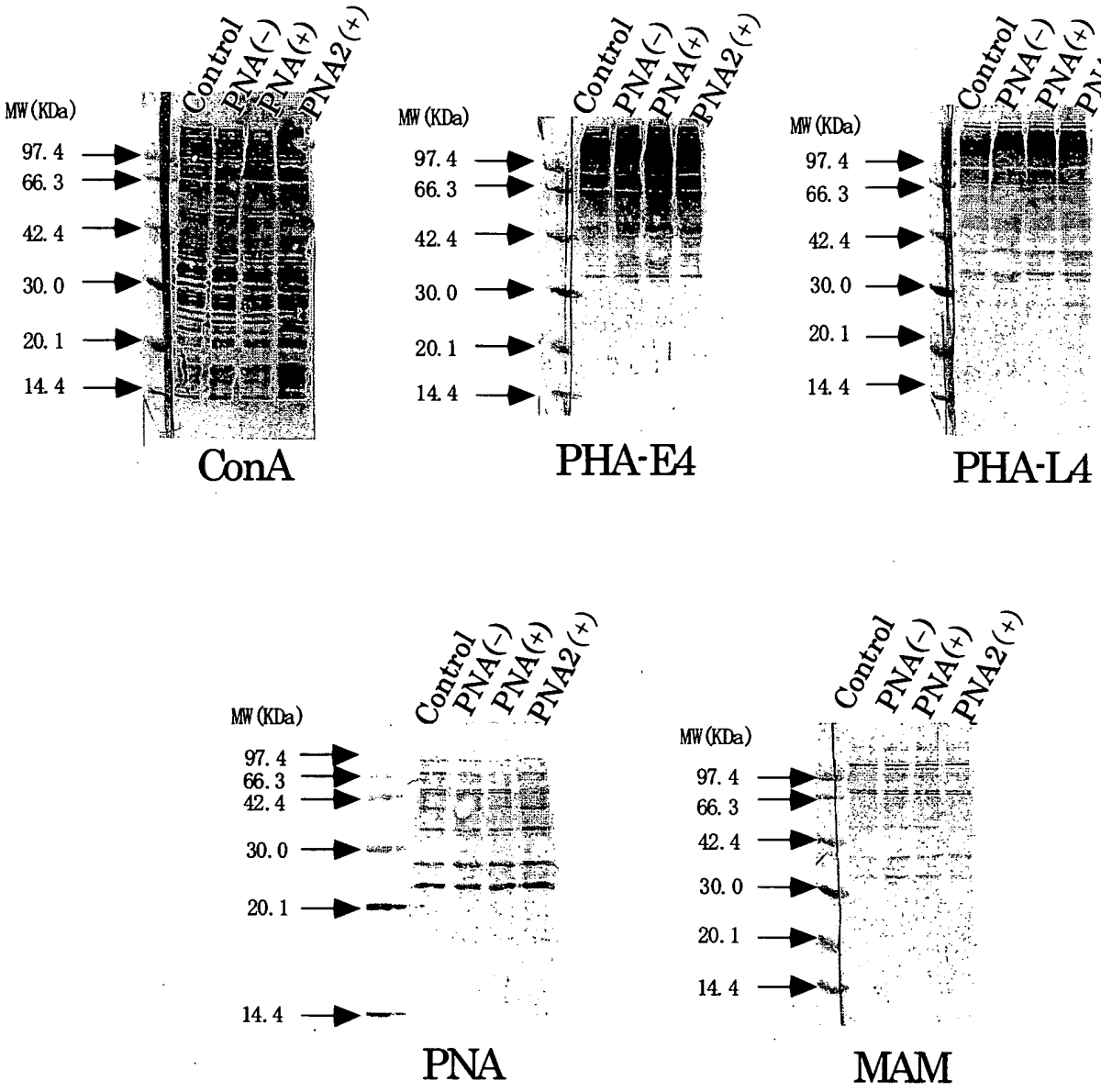
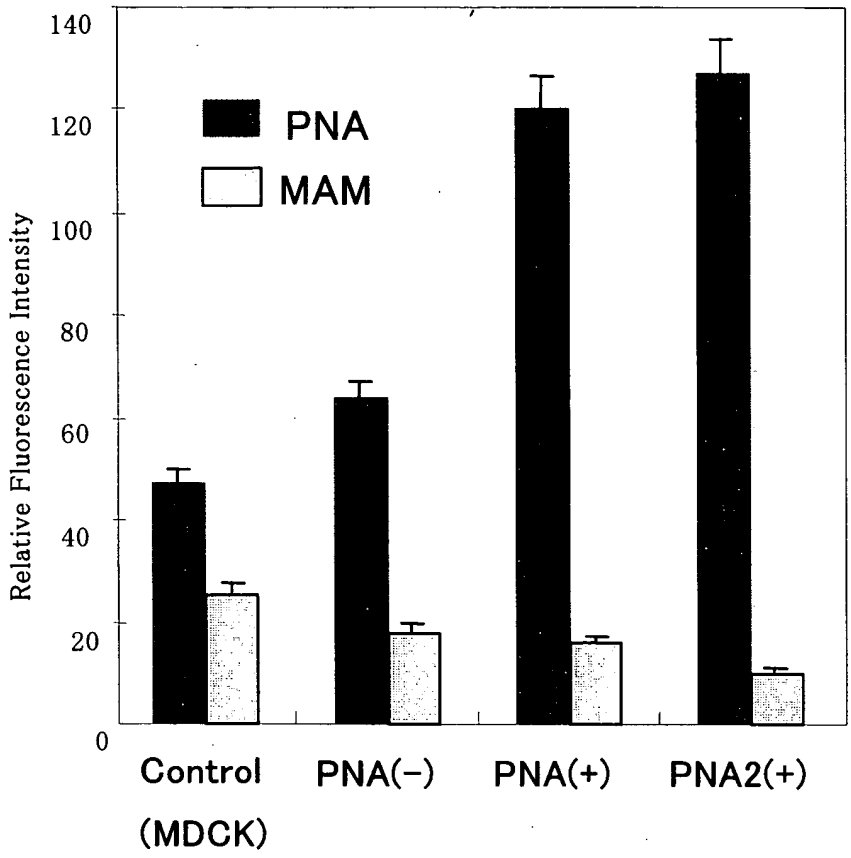

Control
PNA(-)
PNA(+)
PNA2(+)
MW(KDa)
97.4
66.3
42.4
30.0
20.1
14.4
ConA
PHA-E4
PHA-L4
PNA
MAM

FIG 13


140
120
100
80
60
40
20
0
Relative Fluorescence Intensity
PNA
MAM
Control
(MDCK)
PNA(−)
PNA(+)
PNA2(+)

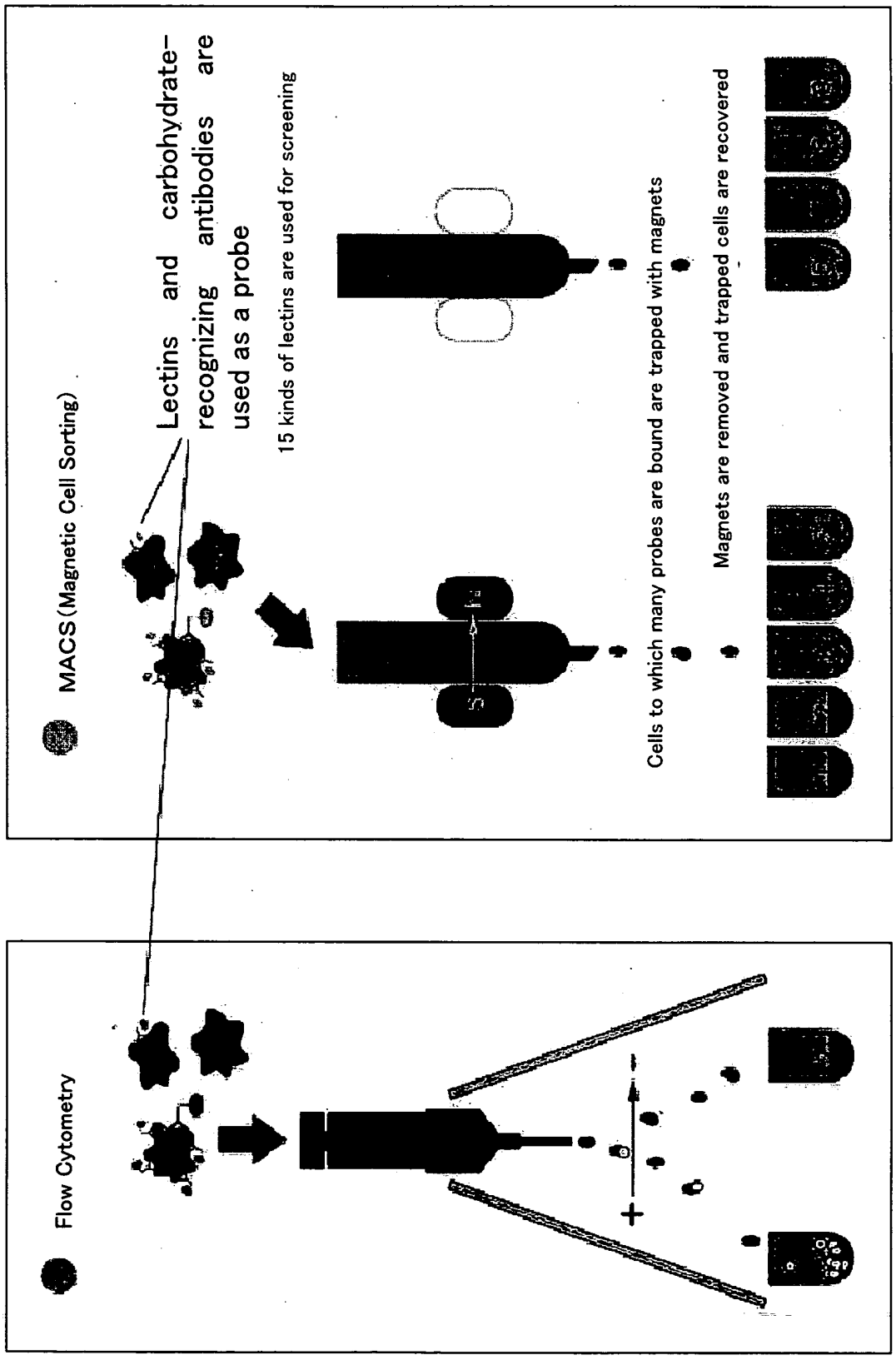

MACS (Magnetic Cell Sorting)
Lectins and carbohydrate-recognizing antibodies are used as a probe
15 kinds of lectins are used for screening
Cells to which many probes are bound are trapped with magnets
Magnets are removed and trapped cells are recovered
Flow Cytometry

FIG 14

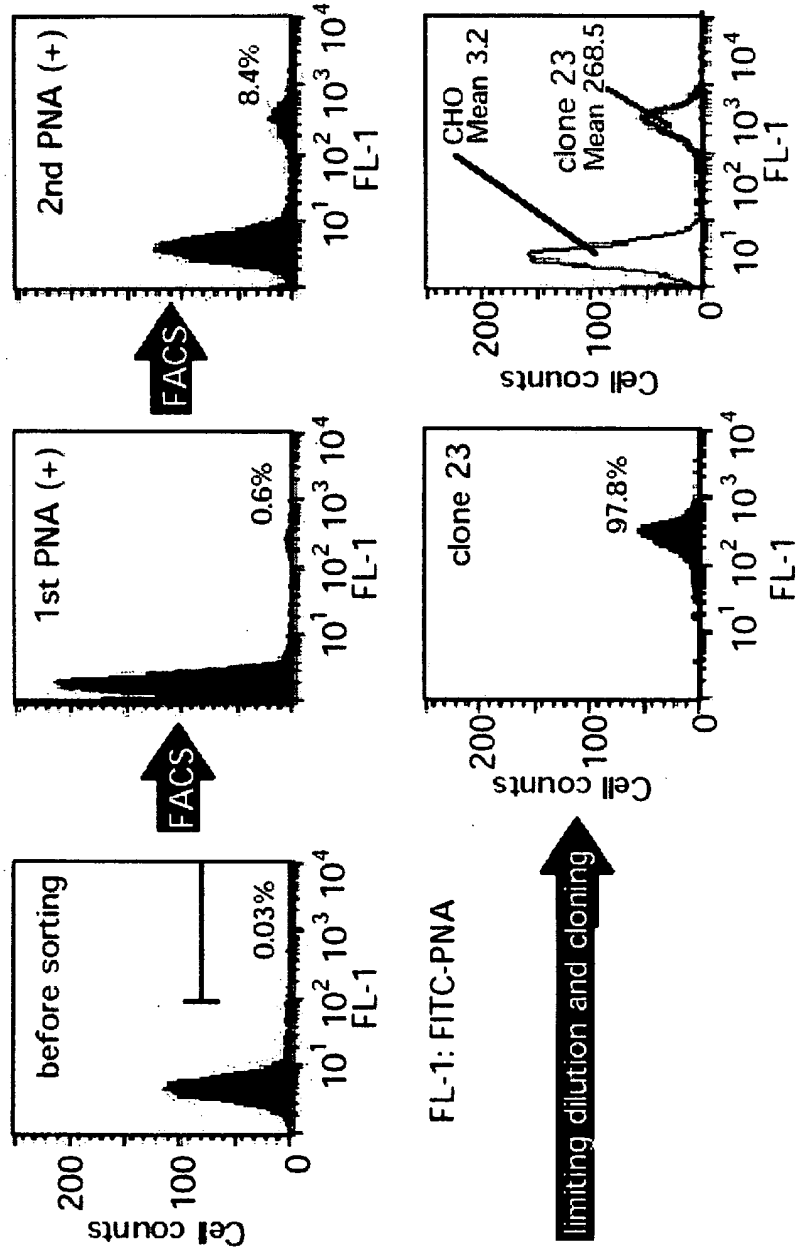

before sorting
0.03%
1st PNA (+)
0.6%
2nd PNA (+)
8.4%
FACS
FACS
Cell counts
200
100
0
FL-1
clone 23
97.8%
CHO
Mean 3.2
clone 23
Mean 268.5
FL-1: FITC-PNA
limiting dilution and cloning

FIG 15

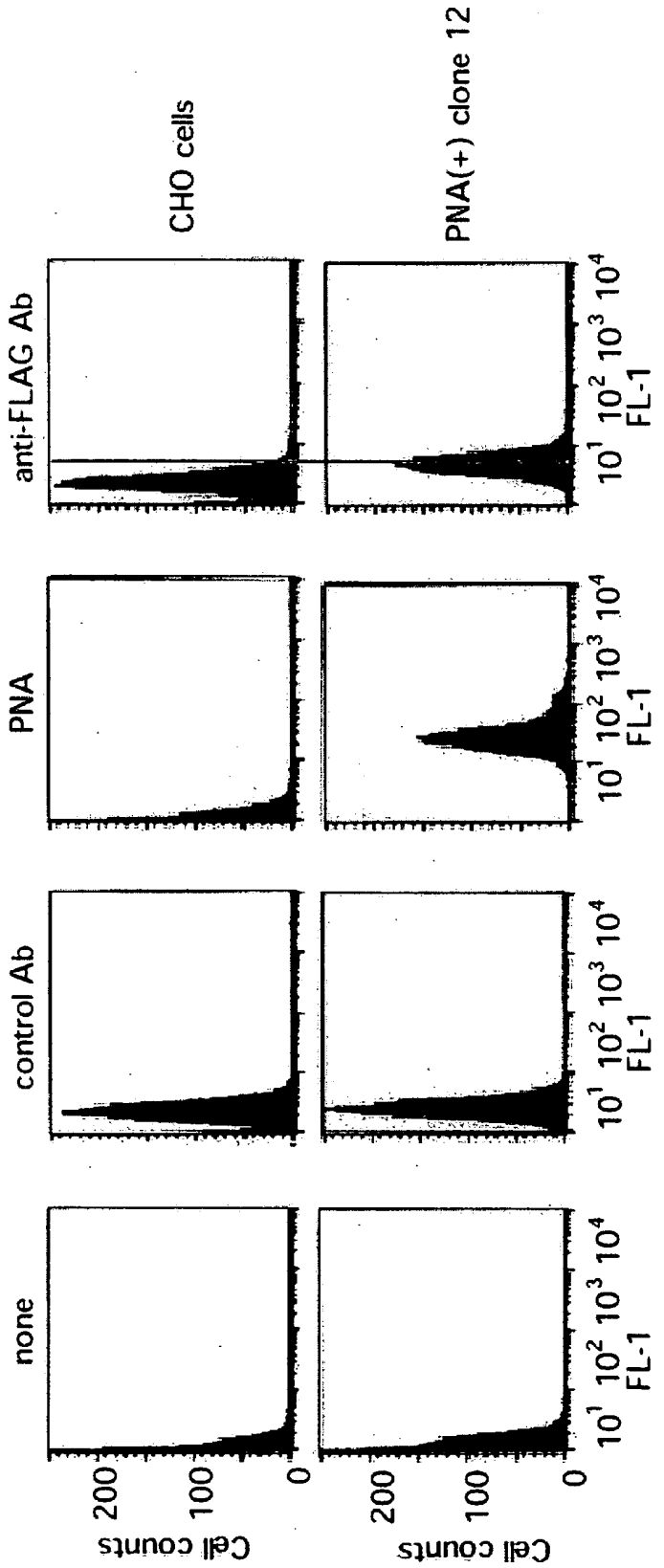

CHO cells
PNA(+) clone 12
anti-FLAG Ab
PNA
control Ab
none
Cell counts
200
100
0
10^1 10^2 10^3 10^4
FL-1

FIG 16